Replication and maintenance of plasmids in Bacillus subtilis

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Chapter III

Effects of the generation of single-stranded DNA on the maintenance of plasmid pMV158 and derivatives in different *Bacillus subtilis* strains

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SUMMARY

The effects of the single-strand origins (SSOs) of plasmid pMV158 on (i) the conversion of its single-stranded (ss) replication intermediates to double-stranded (ds) plasmid DNA and (ii) its maintenance, were analyzed. The rolling-circle plasmid pMV158, which replicates via ssDNA intermediates, contains two single-strand origins (SSOs) of replication, palA and palU. In this chapter the results obtained with Bacillus subtilis are described; complementary studies with Lactococcus lactis are presented in chapter II. While in L.lactis both SSOs are functional as ssDNA conversion signal, only palU appeared to be active B.subtilis. Similar to the situation in L.lactis, the accumulation of large amounts of ssDNA resulted in a severe decrease in plasmid maintenance in B.subtilis. In the latter bacterium large amounts of ssDNA were only accumulated, however, when plasmids lacking a functional SSO were propagated in RecA mutant strains. In wild type RecA strains these plasmids accumulated only modest amounts of ssDNA and they were maintained at fairly stable levels. The results suggest that in B.subtilis a RecA-mediated alternative pathway exists for the conversion of ssDNA which can improve plasmid maintenance. In addition to ssDNA accumulation, and the antagonizing role of RecA therein, two other plasmi regions were shown to affect pMV158 maintenance in B.subtilis. One was mob gene region, which had a negative effect on plasmid maintenance; and the other the palA type SSO. Although palA was not functional as a ssDNA conversion signal in B.subtilis, its presence had a positive effect on pMV158 maintenance.

INTRODUCTION

Most small multicopy plasmids from Gram-positive bacteria replicate via the rolling-circle mechanism (RCM; for reviews see (Gruss and Ehrlich, 1989; Novick, 1989; Jannière et al., 1993), which generates single-stranded plasmid DNA (ssDNA) intermediates. For the efficient initiation of ssDNA conversion to double-stranded (ds) plasmid DNA, single-strand origins (SSOs; formerly referred to as minus origins) are required. Most SSOs that have been identified so far depend on the host-encoded RNA-polymerase to prime complementary DNA strand synthesis on the ssDNA template (Gruss et al., 1987; Boe et al., 1989; Devine et al., 1989; Bron, 1990).

Owing to the fortuitous expression of their natural antibiotic resistance markers in a wide variety of bacteria, many of the cloning vectors used today for Gram-positive bacteria are based on RCM plasmids derived from Staphylococcus aureus. Since SSOs of RCM plasmids are usually only functional in their native host, inefficient ssDNA conversion may occur when vectors based on these plasmids are used in non-native hosts (Gruss et al., 1987; Del Solar et al., 1987; Bron, 1990). This results in the intracellular accumulation of ssDNA intermediates. ssDNA accumulation is frequently associated with a decrease in

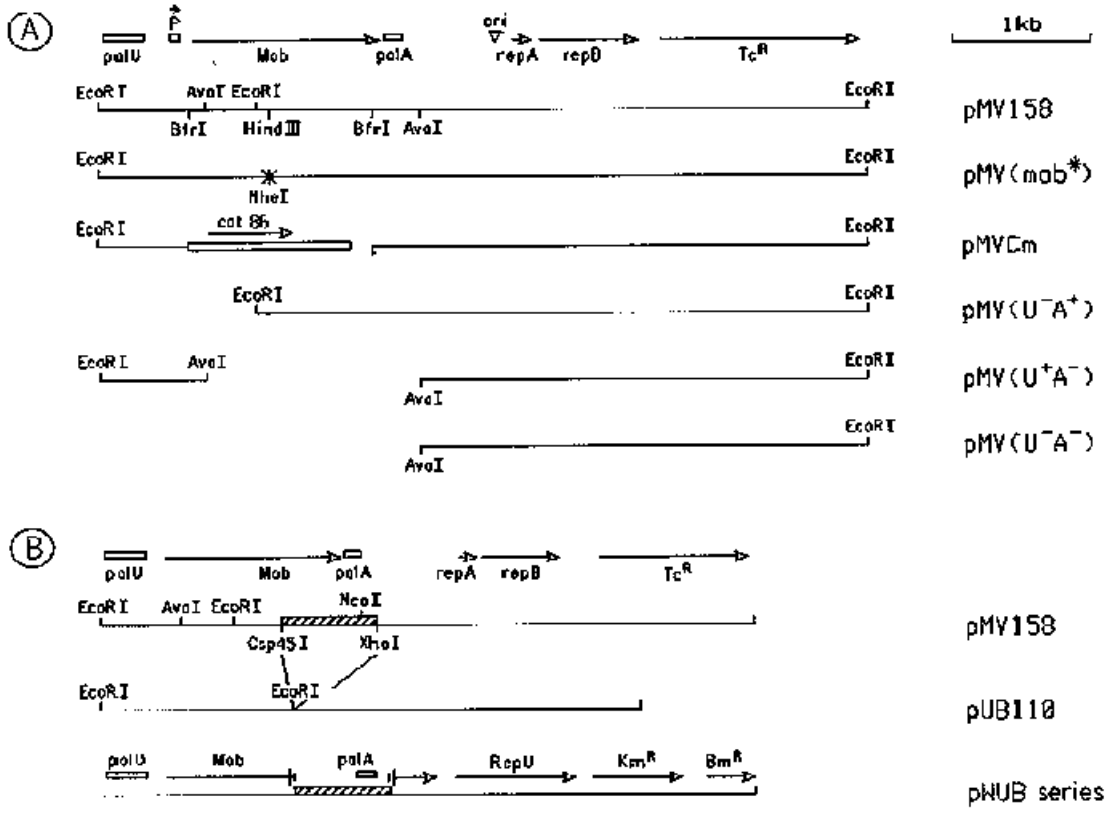

Figure 1. Linear maps of pMV158 and pUB110 derivatives.
(A): pMV158 and derivatives. (B): Derivatives of pUB110 (high- and low-copy number variants) containing palA of pMV158. The positions of palU, the mob gene (Mob) and its promoter (p), palA, the double-strand origin (ori), the replication regions, (repA/repB in pMV158 and repU in pUB110), and the tetracycline, the kanamycin and bleomycin resistance genes (Tet/Tc^R, Km^R, and Bm^R) are indicated. Only restriction sites relevant for constructions are indicated.

plasmid maintenance (Bron et al., 1988a; Bron et al., 1991; Viret and Alonso, 1988).

The aim of the present investigations was to analyze possible causes of poor maintenance of RCM plasmids in Gram-positive bacteria. In particular, we addressed the question whether ssDNA accumulation by the broad-host-range RCM plasmid pMV158 results in decreased plasmid maintenance. pMV158 was originally obtained from Streptococcus agalactiae (Burdett, 1980). In the present chapter the results obtained with Bacillus subtilis are described. Complementary studies with Lactococcus lactis are presented in chapter II.

RESULTS

Construction of pMV158 derivatives

To study the effects of the SSOs and the mob gene of pMV158 on ssDNA accumulation and plasmid maintenance in B.subtilis, several deletion derivatives of pMV158 were constructed (Figs. 1A and 1B). In the nomenclature used for the derivatives the letters "U" and "A"

symbolize the palU and palA-type SSO. The presence or absence of an SSO is indicated by U^+ (or A^+) and U^- (or A^-), respectively. Plasmid pMV(U^-A^+) is identical to the formerly described pLS1 (Lacks et al, 1986). Details of the construction procedures for pMV(U^-A^+), pMV(U^+A^-), pMV(U^-A^-), pMV(mob*) and pMVCm (Fig. 1A) are described in chapter II. In pMV(U^-A^+) the palU SSO and the 5'-part of the mob gene are absent and in pMV(U^+A^-) the palA SSO and the 3'-part of the mob gene were removed. pMV(U^-A^-) lacks both SSOs and the entire mob gene. pMV(mob*) is the derivative in which the pMV158-located mob gene, required for conjugative mobilization of the plasmid (Priebe and Lacks, 1989; van der Lelie et al., 1990), was inactivated by a frameshift mutation. In pMVCm, the mob gene was replaced by the chloramphenicol-resistance (Cm^R) gene of Bacillus pumilus.

pWUB10 and pWUB20 (fig. 1B) are derivatives of the RCM plasmid pUB110 in which the palA SSO of pMV158 was introduced. pUB110 naturally contains palU and a mob gene related to that of pMV158 (van der Lelie et al., 1989). PalA of pMV158 was cloned at the 3'-end of the mob gene of pUB110 to mimic the situation in pMV158. To that aim, pMV158 was digested with Csp45I and XhoI, and pUB110 was linearized with EcoRI. The 5' ends of the purified 813 bp fragment of pMV158, containing palA, and the linear pUB110 were filled-in using Klenow DNA polymerase. After ligation, the mixture was used to transform B.subtilis PSL1 protoplasts. Restriction analysis revealed the presence of palA-containing recombinant plasmids of the expected structure in kanamycin-resistant cells. The pUB110 derivative containing palA in the same orientation relative to the double-strand origin of replication as in pMV158 (the functional orientation) was designated pWUB10, and the derivative with palA in the reversed orientation pWUB20.

Since the copy numbers of these pUB110 derivatives are about 3- to 5-fold higher than those of pMV158, we also constructed low-copy variants of pWUB10 and pWUB20. This was accomplished by exchanging the 3700 bp AccI/BamHI fragment of pWUB10 and pWUB20 by the corresponding fragment from pUB110cop1 (Leonhardt, 1990). The resulting variants, pWUB11 and pWUB21, have copy numbers of about 5 to 10 per chromosome equivalent. pMV(U^-A^-)Em is a spontaneous high-copy-number variant of pMV(UA^-) in which the tetracycline resistance (Tc^R) gene was replaced by the erythromycin resistance (Em^R) gene of pE194. The Tc^R gene was replaced because it was expected that large amounts of the Tc^R gene product, synthesized from the high-copy plasmid, might be toxic to the cells (de la Campa et al., 1990). Sequence-analysis of the high-copy-number mutation revealed the addition of one A-residue at position 658 of pMV(U^-A^+) [coordinates according to (Lacks et al., 1986)]. As a consequence, the gene specifying the RepA protein, which is a repressor for the control of replication (Del Solar et al., 1990; Pérez-Martin and Espinosa, 1991) is out of frame after the third codon. This resulted in a copy number of about 100 per chromosome equivalent.

Effect of the single-strand origins and the Mob protein on plasmid copy numbers

The copy numbers of the different constructs were determined from the ratio of radiolabeled plasmid versus chromosomal DNA. The averages of four independent experiments are listed in Table 1. Except for the high-copy-number mutant pMV(U^-A^-)Em (about 100 copies per chromosome equivalent), all pMV158 derivatives had roughly similar copy numbers in B.subtilis (from about 10 to 17 per chromosome equivalent). The copy numbers of pMV158 variants lacking one or both SSOs were not detectably reduced in

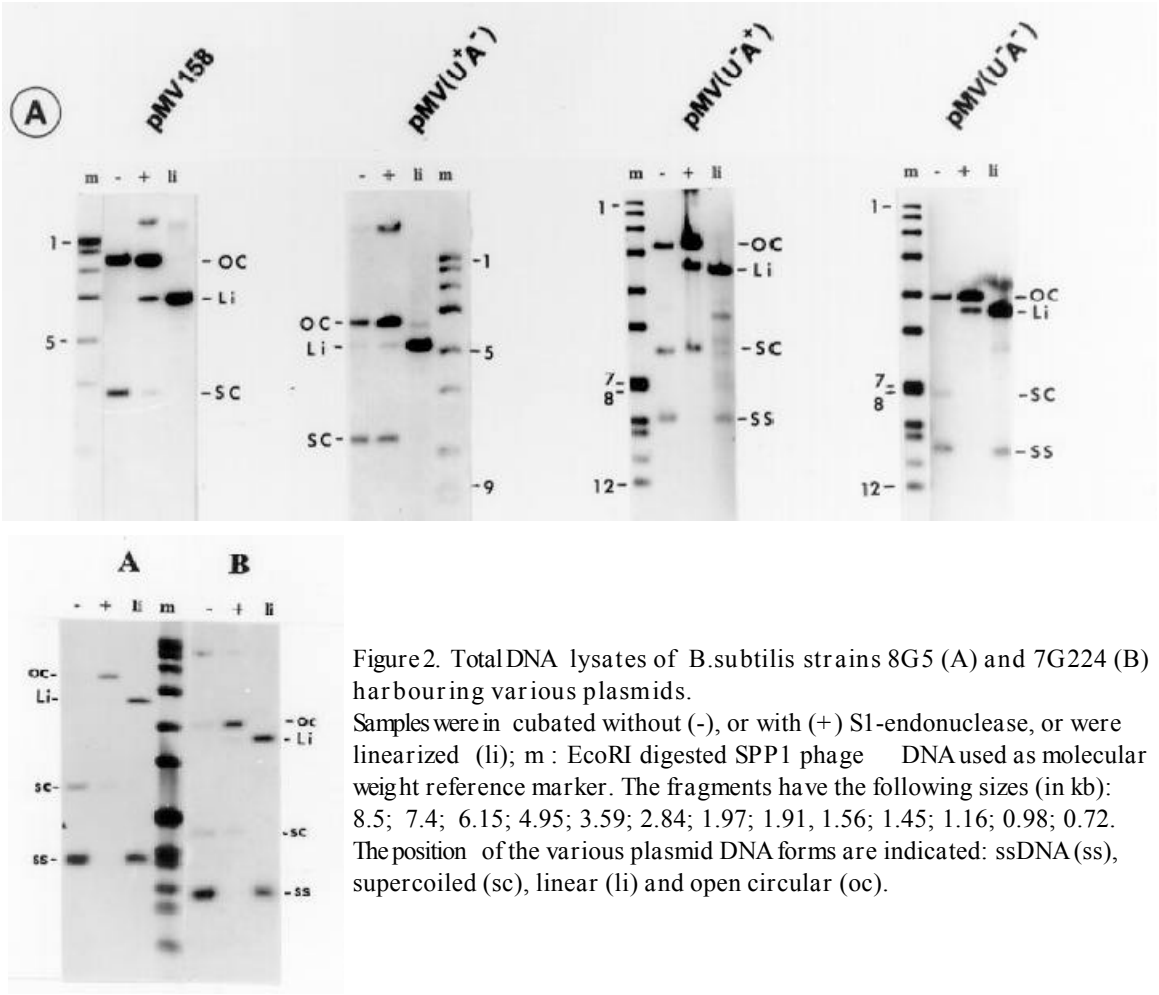

Figure 2. Total DNA lysates of B.subtilis strains 8G5 (A) and 7G224 (B) harbouring various plasmids.
Samples were incubated without (-), or with (+) S1-endonuclease, or were linearized (li); m : EcoRI digested SPP1 phage DNA used as molecular weight reference marker. The fragments have the following sizes (in kb): 8.5; 7.4; 6.15; 4.95; 3.59; 2.84; 1.97; 1.91, 1.56; 1.45; 1.16; 0.98; 0.72. The position of the various plasmid DNA forms are indicated: ssDNA (ss), supercoiled (sc), linear (li) and open circular (oc).

comparison to pMV158.

Effect of the single-strand origins and the mob gene on the accumulation of ss plasmid DNA in different B.subtilis backgrounds

The effects of palU and/or palA, and of the mob gene on the efficiency of conversion of ss to ds plasmid DNA were examined in the B.subtilis strains 8G5 and 7G224. Apart from the recA4 allele, strain 7G224 is isogenic to strain 8G5 which contains the wild-type recA allele (de Vos and Venema, 1981). The recA4 mutation is a point mutation resultin in an inactive RecA protein (de Vos and Venema, 1981). Total DNA extracts from B.subtilis 8G5 cells harbouring pMV158 or its derivatives were divided in two portions, one of which was treated with S1-endonuclease to digest ssDNA; the other portion was left untreated. The results of Southern hybridizations of blots from agarose gels are shown in Fig. 2A. With pMV158 containing both SSOs, no ssDNA could be detected indicating that the conversion of ssDNA was efficient. Inactivation of the mob gene by a frameshift mutation [pMV(mob*)] or the replacement of the entire mob gene by the Cm^R gene did not

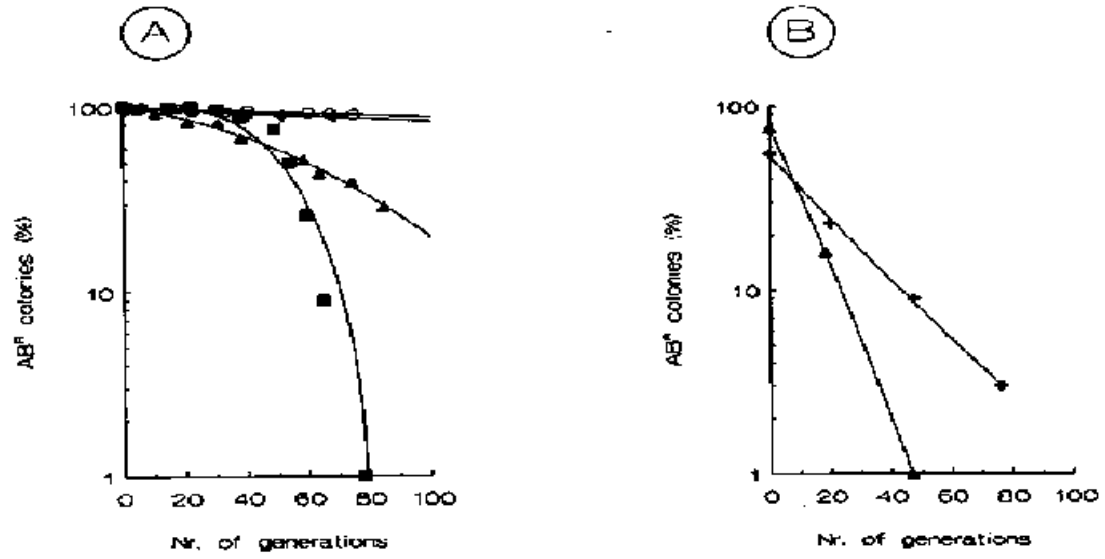

Figure 3. Maintenance of pMV158 and derivatives in the B.subtilis strains 8G5 (A) and 7G224 (B).
#: pMV158; +: pMV(U^-A^+); •: pMV(U^+A^-); >: pMV(U^-A^-).

result in detectable levels of ssDNA accumulation (results not shown). These results indicated that no structural or functional elements from the mob gene region were required for efficient ssDNA conversion.

When only palU was present [pMV(U^+A^-)], no ssDNA was observed under the conditions used. Some ssDNA could be detected, however, when the gels were overloaded (results not shown). With pMV(U^-A^+) ssDNA was observed. Deletion of both SSOs and the mob gene [pMV(U^-A^-)], resulted in about the same level of ssDNA accumulation as when only palA was present. These results indicated that, whereas palU is efficient in B.subtilis, palA has no, or only little activity in this organism. Remarkably, even without a functional ssDNA conversion signal [i.e pMV(U^-A^+) and pMV(U^-A^-)], only modest amounts of ssDNA were detected in strain 8G5, indicating that in these cases ssDNA conversion still occurred rather efficient Strikingly, a large increase in ssDNA accumulation was observed with pMV(U^-A^+) and pMV(U^-A^-) when the DNA extracts were prepared from the recA4 strain 7G224 (Fig. 2B). No differences in ssDNA accumulation between the two strains were observed with constructs containing a functional SSO (i.e. palU; results not shown). These results indicate that RecA can assist in the conversion of ssDNA to dsDNA of pMV158 derivatives devoid of functional SSOs.

Effects of ssDNA accumulation on plasmid maintenance

The possible effects of ssDNA accumulation on the maintenance of the various pMV158 derivatives were measured in B.subtilis 8G5. The results are presented in Fig. 3A. The derivatives containing either palA or palU were maintained quite stably (more than 90% of the cells contained the plasmid after 80 generations of growth in non-selective media). pMV(U^-A^-), lacking both SSOs, was rendered moderately unstable (30% of the cells

contained the plasmid after 80 generations of non-selective growth). Unexpectedly, pMV158, containing both SSOs, was the most unstable of all plasmids tested (less than 5% of the cells harboured the plasmid after 80 generations of growth). The shape of the curve, showing that the kinetics of loss of pMV158 was biphasic, is discussed in one of the following sections. We next studied whether the differences in ssDNA accumulation between the recA wild type and recA4 mutant strains were reflected in differences in plasmid maintenance. As shown in Fig. 3B, the maintenance of pMV(U^-A^-) and pMV(U^-A^+) was strongly decreased in the recA4 strain. Hence, large amounts of ssDNA were paralleled by low levels of plasmid maintenance. In addition, in the wild type recA background, moderate [pMV(U^-A^-)] and low amounts of ssDNA [pMV(U^+A^-)] were paralleled with a moderate and high level of maintenance, respectively.

Maintenance of pMV158 and derivatives was, however, also clearly affected by factors other than ssDNA accumulation. This conclusion is based on the following observations made in the 8G5 recA wild type background:

(a): despite the accumulation of similar amounts of ssDNA with pMV(U^-A^+) and pMV(U^-A^-), the former plasmid was maintained more stably than the latter. Interestingly, the superior maintenance of pMV(U^-A^+) over pMV(U^-A^-) was most pronounced in the recA wild type background (Figs. 3A and 3B).

(b): whereas no ssDNA was detected with pMV158, (containing both SSOs), this plasmid was highly unstable.

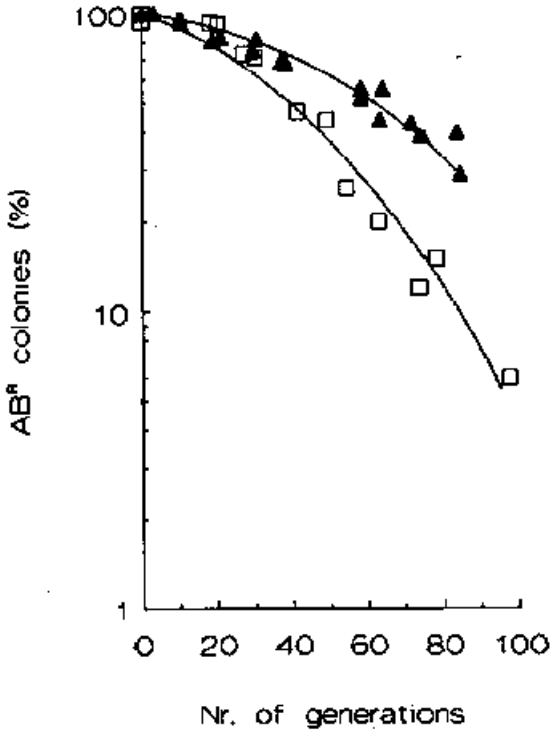

Figure 4. Maintenance of pMV(U^-A^-) and the high-copy number plasmid pMV(U^-A^-)Em in B.subtilis 8G5.
> : pMV(U^-A^-); ***G*** : pMV(U^-A^-)Em

The maintenance of pMV(U^-A^-) is not improved by increasing the copy number

Up to now no active partitioning functions have been identified on RCM plasmids. Therefore, these plasmids are likely to be partitioned randomly and, as a consequence, increased plasmid maintenance is expected with increased copy numbers. To test this prediction, the maintenance of the high-copy-number variant pMV(U^-A^-)Em was compared to that of pMV(U^-A^-) in strain 8G5. Fig. 4 shows that the maintenance of the high-copy-number mutant pMV(U^-A^-)Em was not increased relative to that of pMV(U^-A^-). In fact, the increase in copy number seemed to further decrease the stability of the plasmid.

Sequences within the mob gene underlie the high instability of pMV158 in B.subtilis

Fig. 3A shows that the kinetics of plasmid loss of pMV158 was biphasic Although the onset of the high rate of rapid plasmid loss differed between 30 and 60 generations of growth in independent experiments, the biphasic nature of the kinetics of pMV158 loss was consistently observed. Biphasic segregation kinetics are indicative for the presence of mixed populations of cells, in which one cell type has a growth advantage over the other (Boe et al., 1987). To test whether growth advantages underlie the biphasic nature of the kinetic curves here, we measured the growth rates of plasmid-containing and plasmid-free cells in mixed cultures. Cells containing pMV158, pMV(U^-A^+), pMV(U^+A^-) and pMV(U^-A^-) were used in this test. When mixed in a 1:1 ratio with plasmid-free cells, the frequency of plasmid-containing cells remained approximately constant over 20 generations of growth with pMV(U^-A^+), pMV(U^+A^-) or pMV(U^-A^-). However, with pMV158 the ratio of plasmid-free versus plasmid-containing cells increased from 1 to 185 after 20 generations of growth. Clearly, the presence of pMV158 caused growth retardation to the cells.

Several reasons for the high instability of pMV158 were considered.

(a) To study whether the instability was caused by the presence of the Mob protein, the maintenance of pMV(mob*), producing an inactive Mob protein, was examined pMV(mob*) showed the same low stability as pMV158 (Fig. 5). As with pMV158, cells containing pMV(mob*) were rapidly outgrown by plasmid-free cells (results not shown). Apparently, the low stability of pMV158 was not associated with the presence of functional Mob protein.

(b) To test whether the joint presence of

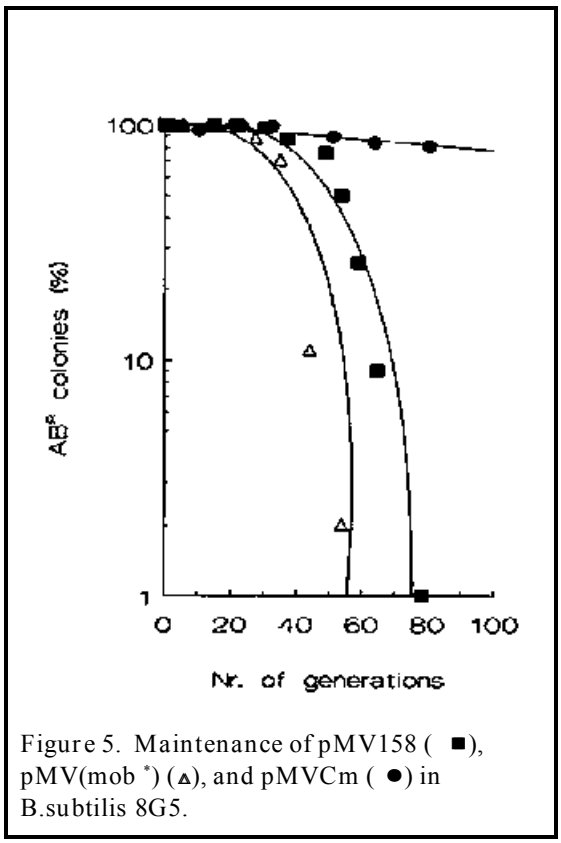

Figure 5. Maintenance of pMV158 (■), pMV(mob *) (▲), and pMVCm (●) in B.subtilis 8G5.

palA and palU caused the high levels of instability of pMV(mob*) and pMV158, the maintenance of pWUB10, -20, -11 and -21 was tested. These plasmids are high-copy number (pWUB10 and pWUB20) and low-copy number (pWUB11 and pWUB21) derivatives of pUB110 which carry the palU SSO (from pUB110) and the palA SSO (from pMV158) at similar positions relative to the mob gene as pMV158. The amino acid sequences of the Mob proteins specified by pMV158 and pUB110 show 45% identity in the N-terminal 200 amino acids (van der Lelie et al., 1989). No plasmid loss was observed with any of the pWUB plasmids during 100 generations of growth (results not shown). This indicated that the joint presence of palA and palU on the same replicon did not cause instability of pUB110 derivatives. We consider it, therefore, also unlikely that the presence of these two SSOs caused the instability of

pMV158 and pMV(mob*) in B.subtilis 8G5. (c) We next tested whether the replacement of the entire mob gene by the Cm^R gene (pMVCm) was able to restore plasmid maintenance. No ssDNA was observed and, in contrast to pMV158 and pMV(mob*), pMVCm was only slightly unstable (more than 80% of the cells contained the plasmid after 80 generations of growth; Fig. 5) These results indicate that the high instability of pMV158 and pMV(mob*) was caused by either DNA sequences within the mob gene, or by the N-terminal part of the Mob protein which was not affected by the mutation in plasmid pMV(mob*).

DISCUSSION

A major conclusion from the present work is that several factors affect the maintenance of the rolling-circle, broad-host-range plasmid pMV158 in B.subtilis. First, the accumulation of high amounts of ssDNA was associated with severe plasmid losses. Second, the palA-type SSO, although not an effective ssDNA conversion signal, had a positive effect on plasmid maintenance. Third, sequences within the mob gene or part of the Mob protein interfered with plasmid maintenance. Another important conclusion is that the host-encoded RecA protein drastically reduces ssDNA accumulation of pMV158 derivatives lacking a functional SSO and thereby improves plasmid maintenance.

Effects of the pMV158 SSOs on ssDNA accumulation and plasmid maintenance

The absence of a functional SSO from RCM plasmids results in the accumulation of ssDNA (Del Solar et al, 1987; Boe et al., 1989; Gruss and Ehrlich, 1989; Bron, 1990; Bron et al., 1991). In L.lactis, the accumulation of large amounts of ssDNA appeared to be a major factor causing a decrease in the maintenance of pMV158 derivatives (Meijer et al., 1995) The present work showed that also in B.subtilis large amounts of ssDNA were paralleled by a strong decrease in the maintenance of pMV158 derivatives. These results confirm earlier observations that accumulation of ss replication intermediates of RCM plasmids are associated with decreased plasmid maintenance in Gram-positive bacteria (Bron et al., 1987; Bron et al., 1988a; Bron et al., 1991; Gruss et al., 1987; Del Solar et al., 1987; Gruss and Ehrlich, 1989).

Alternative, RecA-dependent pathway for ssDNA conversion

The present results also showed that plasmids lacking a functional SSO accumulated much more ssDNA in B.subtilis recA4 mutants than in wild-type recA strains. This suggests that the B.subtilis RecA protein is involved in an alternative, SSO-independent pathway of ssDNA conversion. Since the maintenance of plasmids lacking a functional SSO was much higher in the wild type recA background than in the recA4 mutant, this alternative ssDNA conversion pathway seems to be of considerable importance for the maintenance of, at least, pMV158 derivatives. Recently, we have obtained evidence that, in addition to the host-encoded RecA protein, also the plasmid region upstream of the double-strand origin (plus origin), which specifies an RNA transcript complementary to the displaced ssDNA strand, is important for the alternative ssDNA conversion pathway. We hypothesize that the RecA protein stimulates the annealing of the RNA transcript to the displaced leading strand during rolling-circle replication. The annealed transcript can then serve as a primer for lagging strand synthesis Detailed analyses of this alternative ssDNA conversion route is described in chapter IV. The involvement of host-recombination

enzymes in plasmid maintenance has been reported before (Alonso et al., 1987; Viret and Alonso, 1988). These authors showed that pC194, lacking an SSO which is functional in B.subtilis, is unstable in a B.subtilis recA4 background but not in a wild type recA strain. These authors also showed that pUB110, containing an SSO which is functional in B.subtilis, was maintained stably in both backgrounds (Alonso et al., 1987).

Positive effects of the palA SSO on plasmid maintenance

Although pMV(U^-A^+) and pMV(U^-A^-) generated similar amounts of ssDNA, the former was maintained markedly better than the latter in a wild-type recA background. Since the major difference between these plasmids is palA, this SSO, which is not functional as an ssDNA conversion signal in B.subtilis, is apparently involved in the maintenance of pMV158. Del Solar et al. (1993) have recently identified stretches of homology between part of palA and the partition (par) region of the E.coli plasmid pSC101. The highest homology was found with the region containing the DNA gyrase binding site within this par site (Wahle and Kornberg, 1988). Possibly, host-encoded enzymes, such as DNA gyrase, bind to the palA region, thus affecting plasmid supercoiling and, as a consequence, plasmid maintenance (Del Solar et al., 1993). That altered levels of supercoiling can affect plasmid maintenance is known from several studies with E.coli (Beaucage et al., 1991). A stabilizing effect of palA was also observed in S.pneumoniae (Del Solar et al., 1993). Therefore, it is likely that palA, besides its ssDNA conversion activity in some Gram-positive bacteria, additionally can increase plasmid maintenance through, most likely, alterations in plasmid configuration. However, since pMV(U^-A^+) is unstably maintained in B.subtilis recA4 strains, we conceive that the stabilizing effect of palA is masked by the destabilizing effects caused by the large amounts of ssDNA accumulated in these strains.

Sequences within the pMV158 mob gene interfere with plasmid maintenance

pMV158, containing both SSOs, was maintained stably in L.lactis (see chapter II) but not in B.subtilis. Since with pMV158 no ssDNA was detectable, the observed instability in B.subtilis must be a consequence of other factors. We could exclude the possibilities that the presence of an intact Mob protein, or the joint presence of palA and palU on the same plasmid, interfered with plasmid maintenance. The results indicated that B.subtilis cells containing pMV158 suffered from a severe growth disadvantage, resulting in a rapid outgrowth of pMV158-containing cells by their plasmid-free progeny. Although we cannot entirely rule out the possiblity that the high instability is caused by expression of part of the Mob protein [as pMV(mob*) was also highly unstable], the observation that replacement of the pMV158 mob gene by other DNA sequences resulted in a drastic improvement of plasmid maintenance most probably indicates that sequences within the mob gene were responsible for the high instability of pMV158. So far, we have not delineated the sequences within mob causing this instability in B.subtilis. However, the effect cannot be caused by promoter activity of the mob gene, since this region is still intact in pMV(U^+A^-) and this plasmid is maintained stably. Although the mechanism causing the instability of pMV158 in B.subtilis cannot be precisely defined at present, it is clear that it is host-dependent and that the type of mob gene is crucial, as pUB110 carrying a related mob gene, is stably maintained in B.subtilis.

Taken together, the results described in this chapter and those described in chapter II showed that, in addition to

several other factors, the accumulation of large amounts of ssDNA strongly interfered with stable plasmid maintenance in both bacteria tested (L.lactis and B.subtilis). Since it is not precisely known how ssDNA accumulation leads to plasmid loss, we can only speculate about possible reasons for this correlation.

(a) It has been reported that the absence of a functional SSO from certain plasmids in S.aureus (Gruss et al., 1987) and S.pneumoniae (Del Solar et al., 1987) results in a reduction in plasmid copy number, which could be the cause of the observed decrease in plamid maintenance in those cases. However, such an explanation is unlikely to account for the observed decrease in plasmid maintenance in our studies. First, no reduction in copy number was observed, and second, maintenance of the high-copy-number plasmid pMV(U$^-$A$^-$)Em was not improved compared to pMV(U$^-$A$^-$).

(b) It is conceivable that ssDNA accumulation results in, or increases the size of, subpopulations of cells within the culture that have a higher than average probability of plasmid loss. This would be the case if this subpopulation consists of cells having a lower than average number of segregating plasmid units, for instance, due to a higher spread in plasmid copy numbers. In this respect the recent work of Tolker-Nielsen and Boe (1994) is of interest. These authours performed statistical analyses on the formation of plasmid-free cells in E.coli populations harbouring pBR322-derived plasmids. A major conclusion was that the kinetics of plasmid loss did not fit into the conventional mathematical models due to the presence of subpopulations of plasmid-containing cells which gave rise to progeny that produced plasmid-free cells with a high and unpredictable rate. So far, analyses of a possible effect of ssDNA accumulation on the variation of plasmid copy numbers in individual cells have not been published.

(c) ssDNA accumulation might affect the growth rate of the cells, which consequently results in rapid outgrowth of plasmid-containing cells by plasmid-free cells. The obtained results indicate that this is a likely explanation for the observed instability of pMV(U$^-$A$^-$) in L.lactis (see chapter II).

MATERIALS AND METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used are listed in Table 1. TY medium, used for culturing Escherichia coli and B.subtilis, contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). TY plates contained in addition 2% agar. Tetracycline, kanamycin and chloramphenicol were added to final concentrations of 10 µg/ml.

DNA techniques. All DNA manipulations, including the restriction of DNA, plasmid isolation, preparation of total lysates, purification of DNA fragments from gels, Southern hybridizations and the quantitation of ssDNA, were carried out as described in chapter II.

Transformation of B.subtilis and E.coli. Competent cells and protoplasts of B.subtilis were prepared and transformed as described (Bron, 1990). $CaCl_2$-treated E.coli cells were transformed as described (Sambrook et al., 1989).

Copy number determinations and assays of segregational plasmid stability. These procedures were as described in chapter II. The genome size of B.subtilis was taken to be 4.12×10^6 bp (Amjad et al., 1991).

Growth rates of plasmid-containing and plasmid-free cells. Two cultures of logarithmically growing cells in selective medium, one with cells containing the plasmid and the other with plasmid-free cells, were mixed, and after centrifugation resuspended in non-selective medium. The plasmid-free cells were distinguished from

Table 1. Plasmids and strains

Plasmid	size[a]	Relevant properties	Copy number[b]	Reference
pMV158	5.5	palA, palU, mob-gene, Tc^R	15.1 ± 3.8	Burdett, 1980
pMV(U^-A^+)	4.4	palA, Tc^R	15.7 ± 4.3	Lacks et al., 1986
pMV(U^+A^-)	3.9	palU, Tc^R	17,4 ± 4.9	This study
pMV(mob*)	5.5	palA, palU, inactive Mob, Tc^R	9.6 ± 3.6	This study
pMV(U^-A^-)	3.2	Tc^R	14.6 ± 5.3	This study
pMVCm	5.4	palA, palU, △ mob-gene, Tc^R, Cm^R	15.6 ± 4.2	This study
pMV(U^-A^-)Em	2.8	Em^R	>100	This study
pWUB10	5.4	palA[c], palU, inactive Mob, Km^R	50[e]	This study
pWUB20	5.4	palA[d], palU, inactive Mob, Km^R	50[e]	This study
pWUB11	5.4	palA[c], palU, inactive Mob, Km^R	5[f]	This study
pWUB21	5.4	palA[d], palU, inactive Mob, Km^R	5[f]	This study

Bacterial strain	Genotype	Reference
B.subtilis		
8G5	trpC2 tyr 1 met his ura nic ade rib, wild-type recA	Bron and Venema, 1972
7G224	trpC2 tyr 1 met ura nic ade rib recA4	de Vos and Venema, 1981
E.coli		
JM83	F- ara △(lac-proAB) rpsL (Str^r) (Φ80d △(lacZ)M15)	Vieira and Messing, 1982

a: Size in kb; b: Copy number per chromosome equivalent (average of 4 experiments); c: Same

plasmid-containing cells through a chromosomally located kanamycin resistance gene in the former strain. The initial ratio between plasmid-free and plasmid-containing cells was determined by plating samples on non-selective media directly after mixing the cultures and subsequent replica plating of the resulting colonies on Km- and Tc-containing plates. During growth of the mixed cultures, the three possible cell types were distinguished by plating samples on non-selective plates, and replica plating the resulting colonies onto Km- and Tc-containing plates. To

maintain logarithmic growth of the cells the mixed cultures were diluted in fresh, prewarmed, non-selective medium after every four hours.

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